BASIC LABORATORY PROTOCOLS

FOR THE PRESUMPTIVE IDENTIFICATION OF

Francisella tularensis

CDC

Centers for Disease Control and Prevention

This protocol is designed to provide laboratories
with techniques to identify microorganisms, in order to
support clinicians in their diagnosis of potential diseases.

Credits

Subject Matter Expert:

May C. Chu, Ph.D.
Chief, Diagnostic and Reference Section
Bacterial Zoonoses Branch
Division of Vector-Borne Infectious Diseases
National Center for Infectious Diseases
Centers for Disease Control and Prevention

Acknowledgments:

Thomas J. Quan, Ph.D.
Retired Chief, Diagnostic and Reference Section
Bacterial Zoonoses Branch

as well as:
Zenda L. Berrada, Holly B. Bratcher, Leon G. Carter,
Devin W. Close, Katie L. Davis, Todd S. Deppe,
Kiyotaka R. Tsuchiya, Betty A. Wilmoth, Brook M. Yockey
and David T. Dennis

Technical Editors:

Kimberly Quinlan Lindsey, Ph.D.
Laboratory Education and Training Coordinator
Bioterrorism Preparedness and Response Program
Centers for Disease Control and Prevention

Stephen A. Morse, M.S.P.H., Ph.D.
Deputy Director Laboratory Services
Bioterrorism Preparedness and Response Program
Centers for Disease Control and Prevention

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I. Introduction

1. Disease History and Perspective

A plague-like disease in California ground squirrels was described by McCoy in 1911. The causative agent was named *Bacterium tularense* (McCoy and Chapin, 1912). The human disease was recognized and described by Edward Francis (Francis, 1921) as tularemia, and the agent was renamed *Francisella tularensis* in his honor. Tularemia is a disease of wild animals. Ticks, mosquitoes and biting flies have been implicated as vectors of tularemia bacteria that infect animals and humans. Contaminated hay, water, infected carcasses, chronically infected animals and aerosolized particles have been documented as sources of infection. *F. tularensis* is one of the most infectious bacteria known and can cause severe illness and death in humans (Overholt *et al.*, 1961; Taylor *et al.*, 1991). Thus, it is considered an important potential weapon for bioterrorism.

2. Epidemiology

Tularemia remains widely enzootic in North America, Europe and northern Asia. Humans acquire infection by inadvertent exposure from the bite of an infected vector, or by handling, ingesting, or inhaling infectious materials. Human cases typically are sporadic, but outbreaks do occur. Since 1950, the incidence of reported cases in the United States has steadily declined. In 1995, the Council of State and Territorial Epidemiologists (CSTE) elected to remove tularemia from the list of nationally notifiable disease effective January 1, 1996. Thirty-six states continue to include tularemia on their list of reportable diseases. CDC has made a request to CSTE to reinstate tularemia as a reportable disease by Year 2000.
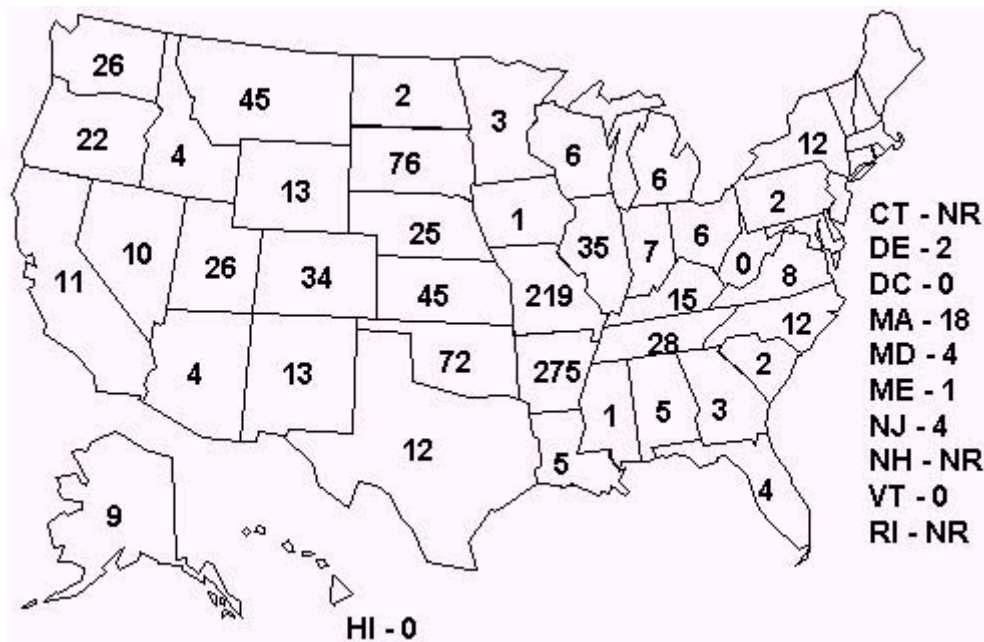

Figure 1. Reported cases of tularemia, 1990-1998

3. Infection and disease

Tularemia is a plague-like disease in humans. It is frequently misdiagnosed early in infection since its symptoms are not unique: sudden onset of chills, fever, headache and generalized malaise. The incubation period is 2-10 days. The bacteria replicate in the skin at the localized site of penetration where an ulcer usually forms. From the penetration site(s) bacteria are transported by the lymphatic system to regional nodes and then may be disseminated to other sites if the infection has not abated. Tularemia presents in humans primarily as an ulceroglandular disease (45%-80% of the reported cases), as glandular infection (10%-25%) and less frequently, as oculoglandular (<5%), septic (<5%), oropharyngeal (<5%) and pneumonic (<5%) forms. Onset is sudden: typically, the patient has a temperature of 38° C -40° C accompanied by chills, headache, generalized body aches (often prominent in the low back), coryza, pharyngitis, cough, and chest pain or tightness. Without treatment, nonspecific symptoms usually persist for several weeks, and sweats, chills, progressive weakness, and weight loss characterize the illness. Any of the principal forms of tularemia may be complicated by bacteremic spread, leading variously to tularemic pneumonia (common), sepsis (uncommon), and meningitis (rare).

4. Bacteriologic characteristics

F. tularensis is a tiny, pleomorphic, gram-negative, facultative intracellular coccobacillus (0.2-0.5 μm X 0.7-1.0 μm). It is a fastidious organism and usually requires cysteine supplementation for good growth on general laboratory media*. F. tularensis* is non-motile and has a thin capsule consisting of lipids, proteins and carbohydrates. Biochemically, it is fairly inert, using glucose as the carbon source.

5. Treatment and prevention

F. tularensis infections are treatable with first-generation antibiotics; and antibiotic resistance in wild-type isolates is rare. Appropriate and early treatment is effective (Sawyer *et al.*, 1966). Streptomycin is the drug of choice. Doxycycline or chloramphenicol are acceptable alternatives, but occasional treatment failures or relapses may occur with their use. A small number of patients have been successfully treated with ciprofloxacin, suggesting that fluoroquinolones may be useful in treatment. Penicillins and cephalosporins are not effective and should not be used to treat tularemia.

A live attenuated vaccine (LVS) has been in use since 1959 for immunizing of personnel who are at risk of laboratory infection (Burke, 1977). The vaccine is administered as a single dose of 0.06 mL by scarification. Antibodies develop within 3 weeks and may persist at reduced levels for months or years. Human volunteer studies have demonstrated partial protection against aerosol challenges after vaccination. A review of vaccination records from 1960-1969 revealed that there were a total of 11 cases of laboratory-acquired tularemia (1 case per 1,000 at risk employee-years; Burke, 1977) in vaccinated personnel.

6. Hospital precautions and environmental decontamination

Standard Precautions only are required for purposes of hospital infection since person-to-person transmission has not been proven. Bodies of patients dying of tularemia should be handled under Standard Precautions. Autopsy procedures likely to cause aerosols, such as cutting of bone, should be avoided or performed under biosafety level III (BSL-3)

conditions. Clothing or linens contaminated with body fluids of patients infected with *F. tularensis* should be disinfected as per routine hospital protocol. The use of a 10% household bleach solution (0.5% hypochlorite) followed by rinse using 70% alcohol is adequate for surface decontamination.

F. tularensis survives for long periods in water, mud, and animal carcasses, and survival is thought to be enhanced by low ambient temperatures. Straw contaminated by excreta of infected rodents can be aerosolized during farming activities and cause outbreaks of human disease. There is, however, scant information on how long artificially dispersed particles would survive in unnatural environments: in urban centers or within buildings. Routine levels of chlorination in municipal water sources prevents contamination.

II. Laboratory Procedures for the identification of *Francisella tularensis*

1. General

The intent of the laboratory procedures presented in this manual is to assist the clinical microbiologist in the examination of a specimen or culture that may contain *Francisella tularensis*. Guidelines are provided to perform simple laboratory tests that will assist the microbiologist to presumptively rule out the presence of *F. tularensis*. These protocols are abstracted from the Centers for Disease Control and Prevention's (CDC) laboratory manual for tularemia diagnostic tests.

Experimentation in the laboratory led McCoy and Chapin, and Francis to an understanding of the organism's virulence, disease pathogenesis in animals and humans, as well as the role of multiple vectors in transmission of the agent. These researchers also reported their findings on bacteria recovery, culture, and serological diagnostic tests. Their findings constitute the basis of today's diagnostic tests for tularemia.

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources, and inclusion does not imply endorsement by the CDC, the U. S. Public Health Service, Department of Health and Human Services, or the other governmental agencies.

2. Laboratory safety

Working with live *F. tularensis* can be hazardous. *F. tularensis* has a long and notorious history of infecting laboratory personnel working with infectious materials (Francis, 1922; Overholt *et al.*, 1961; Jellison, 1972; Burke, 1977). This was especially so before laminar flow biosafety cabinets, vaccination and appropriate antibiotic therapy were available. Laboratory personnel must avoid any activity in the laboratory that puts individuals at risk for infectious exposure, such as activities that might create aerosols or disseminate droplets. As well, personnel should decontaminate laboratory benches after each use, dispose contaminated supplies into their proper receptacles, avoid touching mucosal surfaces with hands (gloved or ungloved), and not eat or drink in the laboratory. Each time before leaving the laboratory, the laboratory coat should be removed, and gloves taken off and reversed, then disposed to a biohazard container; one's hands should be washed before leaving the laboratory.

3. Processing of clinical specimens

Clinical specimens should be handled under BSL-2 conditions and transferred to BSL-3 conditions as soon as *F. tularensis* is suspected. The greatest risk of laboratory-acquired tularemia is by aerosol inhalation when working with infected materials and cultures. The agent is highly virulent and should be handled by trained and vaccinated personnel.

4. Differential tests for the presumptive identification of *F. tularensis*

4.a. <u>Gram stain</u>

4.a.1. Perspective

Direct microscopic examination of specimens and cultures by Gram stain can provide a rapid presumptive diagnosis. Gram stain results, the shape of cell (cocci, bacilli), and the type of cell arrangement (single, chained, clustered) visualized under light microscopy can provide an assessment of what the etiologic agent may be.

4.a.2. Application

Smears may be prepared for staining from cultures, ulcer swabs, affected eyes, tracheo-bronchial washes, lung aspirates, spleen, liver, blood and sputum.

4.a.3. Reagents

Gram stain kit (Fisher Scientific or equivalent supplier)
Control slide (Fisher Scientific or equivalent supplier)

4.a.4. Materials

Microscope slides
Gas or alcohol burner
Staining rack for slides
Microscope with high power and oil immersion objectives

4.a.5. Procedure

Prepare a thin smear from the specimen on the slide. Let the specimen dry. Heat-fix the smear by passing the slide quickly through a flame; this process kills the bacterial cells and binds the bacterial proteins to the slide. Proceed with staining following kit instructions, or individual laboratory standard protocol. Air dry; examine the smear by using the oil immersion objective on the microscope (800-1000X magnification). The slide may be stored at ambient room temperature after removing the immersion oil, or discarded into a disposal receptacle for glass.

4.a.6. Interpretation

The presence of tiny, 0.2-µm-0.5-µm X 0.7-1.0 µm, pleomorphic, poorly staining gram-negative (pink) rods seen mostly as single cells (Figure 2), along with clinical symptoms compatible with tularemia, suggests that the specimen may contain *F. tularensis.* Efforts should be made to isolate *F. tularensis* by inoculating the specimen into broth tubes and onto agar plates.
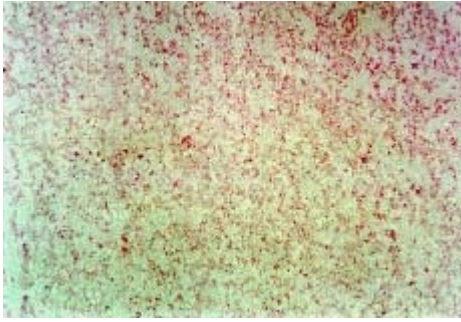
Figure 2. Gram stain of mouse liver tissue infected with *F. tularensis*, 800X

4.a.7. Quality control

Gram staining of test specimens should be carried out with parallel staining of known gram-positive (e.g., *Staphylococcus aureus* or *Bacillus subtilis*) and gram-negative (e.g., *Escherichia coli* or *Klebsiella pneumoniae*) organisms to ensure proper staining results. If this is a frequently performed procedure in the laboratory, parallel staining with controls once a week would be adequate. Reagents, such as iodine and alcohol, should be fresh and the crystal violet stain should be kept free of yeast contamination by filtration. The microscope lens and objectives should be kept dust-free and oil-free. The microscope light source should be aligned to deliver the fullest light. The microscope mirrors should be free of dust and fungus growth.

4.b. Growth of *F. tularensis* in broth

4.b.1. Perspective

Clinical laboratories routinely inoculate clinical specimens in broth bottles for the recovery of the etiologic agent. The most common broths used are brain heart infusion (BHI), trypticase soy broth (TSB) and, sometimes for recovery of more fastidious organisms, thioglycollate broth. *F. tularensis* usually requires enriched medium for growth, especially supplementation with a cysteine-rich source. Specimens suspected to contain live *F. tularensis* cells should be directly inoculated into supplemented broth so that the chance of recovery of this organism is enhanced.

4.b.2. Application

Broth inoculation is not optimal for isolating *F. tularensis*, since it does not grow well in liquid medium. Broth should be used only if a compromised specimen is received and needs to be held for at least 10 days or more to give the agent a chance to revive. Clinical specimens from patients with suspected tularemia should be inoculated on agar or thioglycollate broth (contains cysteine).

4.b.3. Reagents

Clinical specimen or suspect culture.
Brain heart infusion (BHI) broth or trypticase soy broth (TSB), in 5 to 10-ml tubes or in 100-ml culture bottles (Difco Laboratories or equivalent supplier).
Thioglycollate broth, in 5 to 10-ml tubes or in 100-ml culture bottles (Difco or equivalent).
IsoVitaleX, enrichment (Becton Dickinson [BD] Microbiology Systems)

4.b.4. Materials

Incubator, 37° C
Bacteriologic loops, sterile
Test tubes, 1.5-ml sterile
Mortar and pestle, sterile
Sand or latex, sterile
Vortex unit

4.b.5. Procedure

Processing specimens (specimens are to be inoculated simultaneously into broth, agar plates, and other tests if necessary):

(a) For liquid clinical specimens: directly inoculate a loopful into broth. Cap tightly and vortex to mix well.

(b) For viscous materials or skin/wound scrapings: make a suspension of the specimen by mixing it with 0.5-1.0 ml of sterile broth in a tube. Cap the tube tightly and vortex. Transfer up to 0.1 ml of the suspension into a broth for culture. Use the remainder for inoculation onto agar plates and for preparation of smear for staining. Incubate inoculated tube/bottle at 37° C. Observe daily for 10 days for evidence of growth. When automated systems are used, where the culture bottles are shaken, growth may be detected a few days earlier (day 3-7). If turbidity is present, a sample should be removed for sub-culture on appropriate agar and for Gram-staining.
Decontaminate bottles by autoclaving before discarding.

4.b.6. Interpretation

F. tularensis does not grow robustly in broth, even if the medium is supplemented with exogenous cysteine (1% IsoVitaleX). The organism grows slowly and the inoculated broth needs to be observed for at least 10 days. Growth is seen as slight turbidity throughout the medium in BHI (Figure 3a) or TSB. In thioglycollate broth, the growth is seen first as a dense band near the top that diffuses throughout the medium as the culture grows (Figure 3b). Evidence of growth should be followed by Gram staining and by subculture on cysteine-containing agar plates.

Figure 3a. *F. tularensis* SCHU strain inoculated into BHI: left, BHI only; right, BHI + IsoVitalex (cysteine), results are from culture incubated for 48 hours

Figure 3b. *F. tularensis* SCHU strain in thioglycollate broth. From left to right: uninoculated tube, day 10 culture with IsoVitaleX, day 6 and day 10 culture

4.b.7. Quality control

Each lot of broth should be tested by using a panel of control gram-positive/negative strains and a known *F. tularensis* strain to ensure that there are no inhibitory substances that would interfere with growth..

4.c. Growth of *F. tularensis* on agar

4.c.1. Perspective

General microbiological agar such as sheep blood agar (SBA), chocolate agar (CA), and Thayer-Martin agar (TMA) are used for the isolation of bacterial organisms from clinical specimens. *F. tularensis* is fastidious and requires cysteine supplementation for robust growth. *F. tularensis* grows slowly at 35-37° C, and poorly at 28° C; this is a useful feature in distinguishing *F. tularensis* from *Yersinia pestis*. Cysteine heart agar (CHA) supplemented with 9% heated (chocolatized) sheep red blood cells (CHAB) is the preferred medium for growth of *F. tularensis*. This medium provides the necessary nutrition and permits the development of characteristic colonial morphology useful in the diagnosis of *F. tularensis*.

Wild-type *F. tularensis* will grow initially on SBA but will grow poorly or not at all upon subsequent passages. On CA, the colony morphology is bluish white to gray, flat, and dense. Whereas on CHAB, the colonies develop a distinctive sheen and character.

4.c.2. Application

All clinical specimens, tissues recovered from inoculated laboratory mice, or pure cultures are streaked onto agar. If the etiologic agent is unknown, SBA, CA or TMA is inoculated. If the agent is suspected to be tularemia, CHAB plates are inoculated.

4.c.3. Reagents

Sheep blood agar (SBA, 4-6% sheep blood, Becton Dickinson, catalog # 4321239, or other appropriate source).
Chocolate agar (CA, Becton Dickinson, catalog # 4321169, or other appropriate source) and/or Thayer-Martin agar (TMA, Becton Dickinson, catalog # 4321567, or other appropriate source).
Cysteine heart agar (BBL/Difco, catalog # 0047-17-6 powder). Follow manufacturer's directions to make liquid media and supplement with 9% chocolatized sheep blood (CHAB)

4.c.4. Materials

Sterile bacteriologic loops or wood sticks/toothpicks
Incubators: 28° C and 37° C
Microscope, stereoscope 4X magnification
Tape, paper

4.c.5. Procedure

Specimens for culture should be processed simultaneously for growth on agar and for smear.

For cultures, transfer with sterile loop to the agar plates. For tissues, use a sterile wood stick to punch into the tissue several times, especially in visible necrotic areas, and use the stick to inoculate the agar surface. Inoculate two each of SBA and CHAB. If CHAB is not available, use CA.

For safety, tape the top of the petri dish to the bottom in two places to keep them together.

Incubate one each of SBA and CHAB plates at 28° C (to distinguish from *Y. pestis*, *F. tularensis* v. *novicida* and *F. philomiragia* which grows well at this temperature) and another set at 37° C (for development of characteristic colonial morphology). Plates are incubated for at least 24-72 hours and observed daily. If specimens are taken from persons/animals treated with bacteristatic antibiotics, the culture should be held for up to 10 days.

Examine plates for characteristic colonies. After completion of work, decontaminate the plates by autoclaving before discarding the contents.

4.c.6. Interpretation

F. tularensis grows as a grey-white, opaque colony, usually too small to be seen at 24 hours on SBA, CA, TMA, or CHAB. After incubation for 48 hours, colonies on SBA on early passage are about 1-2 mm in diameter, grey-white and opaque. Highly passaged and laboratory-adapted strains do not grow well on SBA (Figure 4a). On TMA and CA surfaces, the colonies are blue-white to grey in color, flat in elevation, with an entire, smooth, and shiny surface (Figure 4b). On CHAB, the growth is more distinct, the colonies are 2-4 mm in size, greenish-white, dense with a butyrous consistency (Figure 4c). A characteristic opalescent sheen is evident on the surface of the colonies, especially if the culture plate has been incubated for 48-72 hours and examined under oblique light. (Figure 4d). There is little or no hemolysis of the sheep red blood cells. The characteristic growth on CHAB suggests that the culture should be further examined by more definitive tests to identify the culture. Forward this culture to the state health department.

Figure 4a. *F. tularensis* SCHU strain on 6% sheep blood agar (SBA), 72 hours. Note its inability to grow well on SBA

Figure 4b. *F. tularensis* SCHU strain on chocolate agar, 72 hours

Figure 4c. *F. tularensis* SCHU strain on cysteine heart agar with 9% chocolatized sheep blood (CHAB), 72 hours

Figure 4d. *F. tularensis* NV972342 isolate on CHAB agar. Opalescent, purplish sheen under oblique light.

4.c.7. Quality control

CHAB can either be purchased commercially or made in the laboratory under aseptic conditions. Each batch of plates must be checked for sterility by incubating plates at 37° C for at least 24 hours. Each batch of plates must also be tested to ensure that it is not inhibitory and will be able to support *F. tularensis* as well as other less fastidious strains (e.g., *S. aureus*, *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*).

5. Actions if a presumptive *F. tularensis* colony is identified and suspected as a bioterrorist threat agent

5.a. Preserve original specimens pursuant to a potential criminal investigation.
5.b. Contact the local FBI, state public health laboratory, and the state public health department.
5.c. Local FBI agents will forward isolates to a state health department laboratory as is necessary. Consultation with a state health department laboratory is strongly encouraged as soon as *F. tularensis* is suspected as a bioterrorist threat agent.

6. Listed vendors

Difco Laboratories, 800 521-0851
Fisher Scientific, 800-766-7000
Becton Dickinson Bioscience [BBL], 800-675-0908

III. References

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IV. Appendix: General overview flowchart

Francisella tularensis Presumptive Identification Flowchart

┌───┐
│ **Suspected Identification of *F. tularensis*** │
│   │
│ Clinical findings, exposure history, and   │
│ compatible Gram stain: (tiny, pleomorphic,   │
│ poorly-staining, gram-negative bacteria)   │
└───┘

⬇
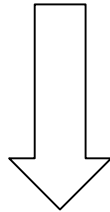
┌───┐
│ **Basis for Presumptive Identification**   │
│ **and Notification of Authorities**   │
│   │
│ Grows poorly on sheep blood agar and at room │
│ temperature   │
│ Distinctive metallic sheen of colonies on cysteine │
│ heart agar with chocolatized blood   │
│   │
│ **Notify** Submitting Agency, Local FBI,   │
│ State Public Health Laboratory, and State Public │
│ Health Department   │
└───┘